

TITLE OF THE INVENTION**IMPROVED SYSTEMS FOR SENSITIVE DETECTION OF G-PROTEIN
COUPLED RECEPTOR AND ORPHAN RECEPTOR FUNCTION
USING REPORTER ENZYME MUTANT COMPLEMENTATION****BACKGROUND OF THE INVENTION**

This application is a continuation-in-part of U.S. Application Serial No.
09/654,499, filed September 1, 2000, which claims the benefit from Provisional
Application Serial No. 60/180,669, filed February 7, 2000. The entirety of U.S.
Application Serial No. 09/654,499 and Provisional Application Serial No.
60/180,669 are incorporated herein by reference.

Field of the Invention

The present invention relates to methods of detecting G-protein-coupled
receptor (GPCR) activity, and provides methods of assaying GPCR activity,
methods for screening for GPCR ligands, agonists and/or antagonists, methods for
screening natural and surrogate ligands for orphan GPCRs, and methods for
screening compounds that interact with components of the GPCR regulatory
process.

Background of the Technology

The actions of many extracellular signals are mediated by the interaction of
G-protein- coupled receptors (GPCRs) and guanine nucleotide-binding regulatory
proteins (G-proteins). G-protein-mediated signaling systems have been identified in
many divergent organisms, such as mammals and yeast. The GPCRs represent a

large super family of proteins which have divergent amino acid sequences, but share common structural features, in particular, the presence of seven transmembrane helical domains. GPCRs respond to, among other extracellular signals, neurotransmitters, hormones, odorants and light. Individual GPCR types activate a particular signal transduction pathway; at least ten different signal transduction pathways are known to be activated via GPCRs. For example, the beta 2-adrenergic receptor (β 2AR) is a prototype mammalian GPCR. In response to agonist binding, β 2AR receptors activate a G-protein (Gs) which in turn stimulates adenylate cyclase activity and results in increased cyclic adenosine monophosphate (cAMP) production in the cell.

The signaling pathway and final cellular response that result from GPCR stimulation depends on the specific class of G-protein with which the particular receptor is coupled (Hamm, "The Many Faces of G-Protein Signaling." J. Biol. Chem., 273:669-672 (1998)). For instance, coupling to the Gs class of G-proteins stimulates cAMP production and activation of the Protein Kinase A and C pathways, whereas coupling to the Gi class of G-proteins down regulates cAMP. Other second messenger systems such as calcium, phospholipase C, and phosphatidylinositol 3 may also be utilized. As a consequence, GPCR signaling events have predominantly been measured via quantification of these second messenger products.

The decrease of a response to a persistent stimulus is a widespread biological phenomenon. Signaling by diverse GPCRs is believed to be terminated by a uniform two-step mechanism. Activated receptor is first phosphorylated by a

GPCR kinase (GRK). An arrestin protein binds to the activated and phosphorylated receptor, thus blocking G-protein interaction. This process is commonly referred to as desensitization, a general mechanism that has been demonstrated in a variety of functionally diverse GPCRs. Arrestin also plays a part in regulating GPCR internalization and resensitization, processes that are heterogenous among different GPCRs (Oakley, et al., J. Biol. Chem., 274:32248-32257 (1999)). The interaction between an arrestin and GPCR in processes of internalization and resensitization is dictated by the specific sequence motif in the carboxyl terminus of a given GPCR. Only a subset of GPCRs, which possess clusters of three serine or threonine residues at the carboxyl termini, were found to co-traffick with the arrestins into the endocytic vesicles after ligand stimulation. The number of receptor kinases and arrestins involved in desensitization of GPCRs is rather limited.

A common feature of GPCR physiology is desensitization and recycling of the receptor through the processes of receptor phosphorylation, endocytosis and dephosphorylation (Ferguson, et al., "G-protein-coupled receptor regulation: role of G-protein-coupled receptor kinases and arrestins." Can. J. Physiol. Pharmacol., 74:1095-1110 (1996)). Ligand-occupied GPCRs can be phosphorylated by two families of serine/threonine kinases, the G-protein-coupled receptor kinases (GRKs) and the second messenger-dependent protein kinases such as protein kinase A and protein kinase C. Phosphorylation by either class of kinases serves to down-regulate the receptor by uncoupling it from its corresponding G-protein. GRK-phosphorylation also serves to down-regulate the receptor by recruitment of a

class of proteins known as the arrestins that bind the cytoplasmic domain of the receptor and promote clustering of the receptor into endocytic vesicles. Once the receptor is endocytosed, it will either be degraded in lysosomes or dephosphorylated and recycled back to the plasma membrane as a fully-functional receptor.

Binding of an arrestin protein to an activated receptor has been documented as a common phenomenon of a variety of GPCRs ranging from rhodopsin to β 2AR to the neurotensin receptor (Barak, et al., "A β -arrestin/Green Fluorescent Fusion Protein Biosensor for Detecting G-Protein-Coupled Receptor Activation," J. Biol. Chem., 272:27497-500 (1997)). Consequently, monitoring arrestin interaction with a specific GPCR can be utilized as a generic tool for measuring GPCR activation. Similarly, a single G-protein and GRK also partner with a variety of receptors (Hamm, et al. (1998) and Pitcher et al., "G-Protein-Coupled Receptor Kinases," Annu. Rev. Biochem., 67:653-92 (1998)), such that these protein/protein interactions may also be monitored to determine receptor activity.

Many therapeutic drugs in use today target GPCRs, as they regulate vital physiological responses, including vasodilation, heart rate, bronchodilation, endocrine secretion and gut peristalsis. See, e.g., Lefkowitz et al., Annu. Rev. Biochem., 52:159 (1983). Some of these drugs mimic the ligand for this receptor. Other drugs act to antagonize the receptor in cases when disease arises from spontaneous activity of the receptor.

Efforts such as the Human Genome Project are identifying new GPCRs ("orphan" receptors) whose physiological roles and ligands are unknown. It is estimated that several thousand GPCRs exist in the human genome.

Various approaches have been used to monitor intracellular activity in response to a stimulant, e.g., enzyme-linked immunosorbent assay (ELISA); Fluorescence Imaging Plate Reader assay (FLIPR™, Molecular Devices Corp., Sunnyvale, CA); EVOscreen™, EVOTEC™, Evotec Biosystems GmbH, Hamburg, Germany; and techniques developed by CELLOMICS™, Cellomics, Inc., Pittsburgh, PA.

Germino et al., "Screening for in vivo protein-protein interactions." Proc. Natl. Acad. Sci., 90(3):933-937 (1993), discloses an *in vivo* approach for the isolation of proteins interacting with a protein of interest.

Phizicky et al., "Protein-protein interactions: methods for detection and analysis." Microbiol. Rev., 59(1): 94-123 (1995), discloses a review of biochemical, molecular biological and genetic methods used to study protein-protein interactions.

Offermanns et al., " $G\alpha_{15}$ and $G\alpha_{16}$ Couple a Wide Variety of Receptors to Phospholipase C." J. Biol. Chem., 270(25):15175-15180 (1995), discloses that $G\alpha_{15}$ and $G\alpha_{16}$ can be activated by a wide variety of G-protein-coupled receptors. The selective coupling of an activated receptor to a distinct pattern of G-proteins is regarded as an important requirement to achieve accurate signal transduction. Id.

Barak et al., "A β -arrestin/Green Fluorescent Protein Biosensor for Detecting G Protein-coupled Receptor Activation." J. Biol. Chem., 272(44):27497-

structural motif on their own; and the use of a combination of the above modifications to achieve even more enhanced detection.

A first aspect of the present invention is a method that monitors GPCR function proximally at the site of receptor activation, thus providing more information for drug discovery purposes due to fewer competing mechanisms.

Activation of the GPCR is measured by a read-out for interaction of the receptor with a regulatory component such as arrestin, G-protein, GRK or other kinases, the binding of which to the receptor is dependent upon agonist occupation of the receptor. The present invention involves the detection of protein/protein interaction by complementation of mutant reporter enzymes.

Binding of arrestin to activated GPCR is a common process in the first step of desensitization that has been demonstrated for most, if not all, GPCRs studied so far. Measurement of GPCR interaction with arrestin via mutant enzyme complementation (i.e., ICAST) provides a more generic assay technology applicable for a wide variety of GPCRs and orphan receptors.

A further aspect of the present invention is a method of assessing GPCR pathway activity under test conditions by providing a test cell that expresses a GPCR, e.g., muscarinic, adrenergic, dopamine, angiotensin or endothelin, as a fusion protein to a mutant reporter enzyme and interacting a protein in the GPCR pathway, e.g., G-protein, arrestin or GRK, as a fusion protein with a complementing mutant reporter enzyme. When test cells are exposed to a known agonist to the target GPCR under test conditions, activation of the GPCR will be

monitored by complementation of the reporter enzyme. Increased reporter enzyme activity reflects interaction of the GPCR with its interacting protein partner.

A further aspect of the present invention is a method of assessing GPCR pathway activity in the presence of a test arrestin, e.g., β -arrestin.

5 A further aspect of the present invention is a method of assessing GPCR pathway activity in the presence of a test G-protein.

A further aspect of the present invention is a method of assessing GPCR pathway activity upon exposure of the test cell to a test ligand.

10 A further aspect of the present invention is a method of assessing GPCR activity upon co-expression in the test cell of a second receptor. The second receptor could be the same GPCR or orphan receptor (i.e., homo-dimerization), a different GPCR or orphan receptor (i.e., hetero-dimerization) or could be a receptor of another type.

15 A further aspect of the present invention is a method for screening for a ligand or agonist to an orphan GPCR. The ligand or agonist could be contained in natural or synthetic libraries or mixtures or could be a physical stimulus. A test cell is provided that expresses the orphan GPCR as a fusion protein with a mutant reporter enzyme, e.g., a β -galactosidase mutant, and, for example, an arrestin or mutant form of arrestin as a fusion protein with a complementing mutant reporter
20 enzyme, e.g., another β -galactosidase mutant. The interaction of the arrestin with the orphan GPCR upon receptor activation is measured by enzymatic activity of the complemented reporter enzyme. The test cell is exposed to a test compound, and an increase in reporter enzyme activity indicates the presence of a ligand or agonist.

A further aspect of the present invention is a method for screening a protein of interest, for example, an arrestin protein (or mutant form of the arrestin protein) for the ability to bind to a phosphorylated, or activated, GPCR. A test cell is provided that expresses a GPCR as a fusion protein with a mutant reporter enzyme, e.g., a β -galactosidase mutant, and contains arrestin (or a mutant form of arrestin) as a fusion protein with a complementing mutant reporter enzyme, e.g., another β -galactosidase mutant. The interaction of arrestin with the GPCR upon receptor activation is measured by enzymatic activity of the complemented reporter enzyme. The test cell is exposed to a known GPCR agonist and then reporter enzyme activity is detected. Increased reporter enzyme activity indicates that the β -arrestin molecule can bind to phosphorylated, or activated, GPCR in the test cell.

A further aspect of the present invention is a method to screen for an agonist to a specific GPCR. The agonist could be contained in natural or synthetic libraries or could be a physical stimulus. A test cell is provided that expresses a GPCR as a fusion protein with a mutant reporter enzyme, e.g., a β -galactosidase mutant, and, for example, an arrestin as a fusion protein with a complementing mutant reporter enzyme, e.g., another β -galactosidase mutant. The interaction of arrestin with the GPCR upon receptor activation is measured by enzymatic activity of the complemented reporter enzyme. The test cell is exposed to a test compound, and an increase in reporter enzyme activity indicates the presence of an agonist. The test cell may express a known GPCR or a variety of known GPCRs, or may express an unknown GPCR or a variety of unknown GPCRs. The GPCR may be, for example, an odorant GPCR or a β AR GPCR.

A further aspect of the present invention is a method for screening a test compound for GPCR antagonist activity. A test cell is provided that expresses a GPCR as a fusion protein with a mutant reporter enzyme, e.g., a β -galactosidase mutant, and, for example, an arrestin as a fusion protein with a complementing mutant reporter enzyme, e.g., another β -galactosidase mutant. The interaction of arrestin with the GPCR upon receptor activation is measured by enzymatic activity of the complemented reporter enzyme. The test cell is exposed to a test compound, and an increase in reporter enzyme activity indicates the presence of an agonist. The cell is exposed to a test compound and to a GPCR agonist, and reporter enzyme activity is detected. When exposure to the agonist occurs at the same time as or subsequent to exposure to the test compound, a decrease in reporter enzyme activity after exposure to the test compound indicates that the test compound has antagonist activity to the GPCR.

A further aspect of the present invention is a method of screening a sample solution for the presence of an agonist, antagonist or ligand to a GPCR. A test cell is provided that expresses GPCR as a fusion protein with a mutant reporter enzyme, e.g., a β -galactosidase mutant, and contains, for example, a β -arrestin as a fusion protein with a complementing reporter, e.g., another β -galactosidase mutant. The test cell is exposed to a sample solution, and reporter enzyme activity is assessed. Changed reporter enzyme activity after exposure to the sample solution indicates the sample solution contains an agonist, antagonist or ligand for a GPCR expressed in the cell.

A further aspect of the present invention is a method of screening a cell for the presence of a GPCR. According to this aspect, an arrestin fusion protein with a mutant reporter enzyme and a GPCR downstream signaling fusion protein with a mutant reporter enzyme are employed to detect GPCR action. A modification of this aspect of the invention can be employed to provide a method of screening a plurality of cells for those cells which contain a GPCR. According to this aspect, a plurality of cells containing a conjugate comprising a β -arrestin protein as a fusion protein with a reporter enzyme are provided; the plurality of cells are exposed to a GPCR agonist; and activity of reporter enzyme activity is detected. An increase in reporter enzymatic activity after exposure to the GPCR agonist indicates β -arrestin protein binding to a GPCR, thereby indicating that the cell contains a GPCR responsive to the GPCR agonist.

A further aspect of the invention is a method for mapping GPCR-mediated signaling pathways. For instance, the system could be utilized to monitor interaction of c-src with β -arrestin-1 upon GPCR activation. Additionally, the system could be used to monitor protein/protein interactions involved in cross-talk between GPCR signaling pathways and other pathways such as that of the receptor tyrosine kinases or Ras/Raf. According to this aspect, a test cell is provided that expresses a GPCR or other related protein with a mutant reporter enzyme, e.g., a β -galactosidase mutant, and contains a protein from another pathway as a fusion protein with a complementing mutant reporter enzyme, e.g., another β -galactosidase mutant. Increased reporter enzymatic activity indicates protein/protein interaction.

A further aspect of the invention is a method for monitoring homo- or hetero- dimerization of GPCRs upon agonist or antagonist stimulation. Increasing evidence indicates that GPCR dimerization is important for biological activity (AbdAlla, et al., "AT1-receptor heterodimers show enhanced G-protein activation and altered receptor sequestration." *Nature*, 407:94-98 (2000); Bockaert, et al., "Molecular tinkering of G protein-coupled receptors: an evolutionary success." *EMBO J.* 18:1723-29 (1999)). Jordan, et al., "G-protein-coupled receptor heterodimerization modulates receptor function." *Nature*, 399:697-700 (1999), demonstrated that two non-functional opioid receptors, κ and δ , heterodimerize to form a functional receptor. Gordon et al., "Dopamine D2 receptor dimers and receptor blocking peptides." *Bioch. Biophys. Res. Commun.* 227:200-204 (1996), showed different pharmacological properties associated with the monomeric and dimeric forms of Dopamine receptor D2. The D2 receptors exist either as monomers that are selective targets for spiperone or as dimer forms that are targets for nemonapride. Herbert, et al., "A peptide derived from a β 2-adrenergic receptor transmembrane domain inhibits both receptor dimerization and activation." *J.B.C.* 271:16384-92 (1996), demonstrated that the agonist stimulation was found to stabilize the dimeric state of the receptor, whereas inverse agonists favored the monomeric form. Indeed, the same study showed that a peptide corresponding to the sixth transmembrane domain of the β 2-adrenergic receptor inhibited both receptor dimerization and activation. Further, Angers et al., Detection of beta-2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer, *Proc. Natl. Acad. Sci. USA*, 97(7):3684-3689, discloses the use of

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β 2-adrenergic receptor fusion proteins (i.e., β 2-adrenergic receptor fused to luciferase and β 2-adrenergic receptor fused to an enhanced red-shifted green fluorescent protein) to study β 2-adrenergic receptor dimerization.

GPCR dimerization in the context of cellular physiology and

5 pharmacology can be monitored in accordance with the invention. For example, β -galactosidase complementation can be measured in test cells that co-express GPCR fusion proteins of β -galactosidase mutant enzymes, e.g., GPCR₁ $\Delta\alpha$ and GPCR₂ $\Delta\omega$ (FIGURE 27). According to this aspect, the interconversion between monomeric to dimeric forms of the GPCRs or orphan receptors can be measured by mutant
10 reporter enzyme complementation. FIGURE 27 illustrates a test cell co-expressing GPCR or an orphan receptor as a fusion protein with $\Delta\alpha$ form of β -galactosidase mutant (e.g., GPCR₁ $\Delta\alpha$), and the same GPCR or orphan receptor as a fusion protein with $\Delta\omega$ form of β -galactosidase mutant (e.g., GPCR₁ $\Delta\omega$). Formation of the GPCR homodimer is reflected by formation of an active enzyme, which can be
15 measured by enzyme activity assays, such as the Gal-Screen™ assay. Similarly, hetero-dimerization between two distinct GPCRs, or two distinct orphan receptors, or between one known GPCR and one orphan receptor can be analyzed in test cells co-expressing two fusion proteins, e.g., GPCR₁ $\Delta\alpha$ and GPCR₂ $\Delta\omega$. The increased β -galactosidase activity indicates that the two receptors can form a heterodimer.

20 A further aspect of the invention is a method of monitoring the interconversion between the monomeric and dimeric form of GPCRs under the influence of agonist or antagonist treatment. The test receptor(s) can be between the same GPCR or orphan receptor (homodimer), or between two distinct GPCRs

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or orphan receptors (heterodimer). The increased β -galactosidase activity after treatment with a compound means that the compound binds to and/or stabilizes the dimeric form of the receptor. The decreased β -galactosidase activity after treatment with a compound means that the compound binds to and/or stabilizes the monomeric form of the receptor.

A further aspect of the invention is a method of screening a cell for the presence of a GPCR responsive to a GPCR agonist. A cell is provided that contains protein partners that interact downstream in the GPCR's pathway. The protein partners are expressed as fusion proteins to the mutant, complementing enzyme and are used to monitor activation of the GPCR. The cell is exposed to a GPCR agonist and then enzymatic activity of the reporter enzyme is detected. Increased reporter enzyme activity indicates that the cell contains a GPCR responsive to the agonist.

The present invention involves the use of a combination of proprietary technologies (including ICASTTM, Intercistronic Complementation Analysis Screening Technology, Gal-ScreenTM, etc.) to monitor protein/protein interactions in GPCR signaling. As disclosed in U.S. Application Serial No. 09/654,499, the method of the invention in part involves using ICASTTM, which in turn involves the use of two inactive β -galactosidase mutants, each of which is fused with one of two interacting target protein pairs, such as a GPCR and an arrestin. The formation of an active β -galactosidase complex is driven by interaction of the target proteins. In this system, β -galactosidase activity can be detected using, e.g., the Gal-ScreenTM assay system, wherein direct cell lysis is combined with rapid

ultrasensitive chemiluminescent detection of β -galactosidase reporter enzyme.

This system uses, e.g., a Galacton-*Star*® chemiluminescent substrate for measurement in a luminometer as a read out of GPCR activity.

FIGURE 23 is a schematic depicting the use of the complementation technology in the method of the present invention. FIGURE 23 shows two inactive β -galactosidase mutants that become active when they are forced together by specific interactions between the fusion partners of an arrestin molecule and an activated GPCR or orphan receptor. This assay technology will be especially useful in high throughput screening assays for ligand fishing for orphan receptors, a process called de-orphaning. As illustrated in FIGURE 28, a β -galactosidase fusion protein of an orphan receptor (e.g., GPCR_{orphan} $\Delta\alpha$) is co-expressed in the test cell with a fusion protein of β -arrestin (e.g., β -Arr $\Delta\omega$). When the test cell is subjected to compounds, which could be natural or synthetic, the increased β -galactosidase activity means the compound is either a natural or surrogate ligand for this GPCR. The same assay system can be used to find drug leads for the new GPCRs. The increased β -galactosidase activity in the test cell after treatment indicates the agonist activity of the compound. The decreased β -galactosidase activity in the test cell indicates antagonist activity or inverse agonist activity of the compound. In addition, the method of the invention could be used to monitor GPCR-mediated signaling pathways via other downstream signaling components such as G-proteins, GRKs or the proto-oncogene c-Src.

The invention is achieved in part by using ICAST™ protein/protein interaction screening to map signaling pathways. This technology is applicable to

a variety of known and unknown GPCRs with diverse functions. They include, but are not limited to, the following sub-families of GPCRs:

(a) receptors that bind to amine-like ligands-Acetylcholine muscarinic receptor (M1 to M5), alpha and beta Adrenoceptors, Dopamine receptors (D1, D2, D3 and D4), Histamine receptors (H1 and H2), Octopamine receptor and Serotonin receptors (5HT1, 5HT2, 5HT4, 5HT5, 5HT6, 5HT7);

(b) receptors that bind to a peptide ligand-Angiotensin receptor, Bombesin receptor, Bradykinin receptor, C-C chemokine receptors (CCR1 to CCR8, and CCR10), C-X-C type Chemokine receptors (CXC-R5), Cholecystokinin type A receptor, CCK type receptors, Endothelin receptor, Neurotesin receptor, FMLP-related receptors, Somatostatin receptors (type 1 to type 5) and Opioid receptors (type D, K, M, X);

(c) receptors that bind to hormone proteins-Follic stimulating hormone receptor, Thyrotrophin receptor and Lutropin-choriogonadotropic hormone receptor;

(d) receptors that bind to neurotransmitters-substance P receptor, Substance K receptor and neuropeptide Y receptor;

(e) Olfactory receptors-Olfactory type 1 to type 11, Gustatory and odorant receptors;

(f) Prostanoid receptors-Prostaglandin E2 (EP1 to EP4 subtypes), Prostacyclin and Thromboxane;

(g) receptors that bind to metabotropic substances-Metabotropic glutamate group I to group III receptors;

(h) receptors that respond to physical stimuli, such as light, or to chemical stimuli, such as taste and smell; and

(i) orphan GPCRs-the natural ligand to the receptor is undefined.

Use of the ICASTTM technology in combination with the invention provides many benefits to the GPCR screening process, including the ability to monitor protein interactions in any sub-cellular compartment-membrane, cytosol and nucleus; the ability to achieve a more physiologically relevant model without requiring protein overexpression; and the ability to achieve a functional assay for receptor binding allowing high information content.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1. Cellular expression levels of β 2 adrenergic receptor (β 2AR) and β -arrestin-2 (β Arr2) in C2 clones. Quantification of β -galactosidase (β -gal) fusion protein was performed using antibodies against β -gal and purified β -gal protein in a titration curve by a standardized ELISA assay. Figure 1A shows expression levels of β 2AR- β gal $\Delta\alpha$ clones (in expression vector pICAST ALC). Figure 1B shows expression levels of β Arr2- β gal $\Delta\omega$ in expression vector pICAST OMC4 for clones 9-3, -7, -9, -10, -19 and -24, or in expression vector pICAST OMN4 for clones 12-4, -9, -16, -18, -22 and -24.

FIGURE 2. Receptor β 2AR activation was measured by agonist-stimulated cAMP production. C2 cells expressing pICAST ALC β 2AR (clone 5) or parental cells were treated with increasing concentrations of (-)-isoproterenol and 0.1mM

IBMX. The quantification of cAMP level was expressed as pmol/well.

FIGURE 3. Interaction of activated receptor β 2AR and arrestin can be measured by β -galactosidase complementation. Figure 3A shows a time course of β -galactosidase activity in response to agonist (-)isoproterenol stimulation in C2 expressing β 2AR- β gal $\Delta\alpha$ (β 2AR alone, in expression vector pICAST ALC), or a pool of doubly transduced C2 co-expressing β 2AR- β gal $\Delta\alpha$ and β Arr2- β gal $\Delta\omega$ (in expression vectors pICAST ALC and pICAST OMC and clones isolated from the same pod (43-1, 43-2, 43-7 and 43-8)). Figure 3B shows a time course of β -galactosidase activity in response to agonist (-)isoproterenol stimulation in C2 cells expressing β 2AR- β gal $\Delta\alpha$ alone (in expression vector pICAST ALC) and C2 clones co-expressing β 2AR- β gal $\Delta\alpha$ and β Arr1- β gal $\Delta\omega$ (in expression vectors ICAST ALC and pICAST OMC).

FIGURE 4. Agonist dose response for interaction of β 2AR and arrestin can be measured by β -galactosidase complementation. Figure 4A shows a dose response to agonists (-)isoproterenol and procaterol in C2 cells co-expressing β 2AR- β gal $\Delta\alpha$ and β Arr2- β gal $\Delta\omega$ fusion constructs. Figure 4B shows a dose response to agonists (-)isoproterenol and procaterol in C2 cells co-expressing β 2AR- β gal $\Delta\alpha$ and β Arr1- β gal $\Delta\omega$ fusion constructs.

FIGURE 5. Antagonist mediated inhibition of receptor activity can be measured by β -galactosidase complementation in cells co-expressing β 2AR- β gal $\Delta\alpha$ and β Arr- β gal $\Delta\omega$. Figure 5A shows specific inhibition with adrenergic

antagonists ICI-118,551 and propranolol of β -galactosidase activity in C2 clones co-expressing β 2AR- β gal $\Delta\alpha$ and β Arr2- β gal $\Delta\omega$ fusion constructs after incubation with agonist (-)isoproterenol. Figure 5B shows specific inhibition of β -galactosidase activity with adrenergic antagonists ICI-118,551 and propranolol in C2 clones co-expressing β 2AR- β gal $\Delta\alpha$ and β Arr1- β gal $\Delta\omega$ fusion constructs in the presence of agonist (-)isoproterenol.

FIGURE 6. C2 cells expressing adenosine receptor A2a show cAMP induction in response to agonist (CGS-21680) treatment. C2 parental cells and C2 cells co-expressing A2aR- β gal $\Delta\alpha$ and β Arr1- β gal $\Delta\omega$ as a pool or as selected clones (47-2 and 47-13) were measured for agonist-induced cAMP response (pmol/well).

FIGURE 7. Agonist stimulated cAMP response in C2 cells co-expressing Dopamine receptor D1 (D1- β gal $\Delta\alpha$) and β -arrestin-2 (β Arr2- β gal $\Delta\omega$). The clone expressing β Arr2- β gal $\Delta\omega$ (Arr2 alone) was used as a negative control in the assay. Cells expressing D1- β gal $\Delta\alpha$ in addition to β Arr2- β gal $\Delta\omega$ responded agonist treatment (3-hydroxytyramine hydrochloride at 3 μ M). D1(PIC2) or D1(PIC3) designate D1 in expression vector pICAST ALC2 or pICAST ALC4, respectively.

FIGURE 8. Variety of mammalian cell lines can be used to generate stable cells for monitoring GPCR and arrestin interactions. FIGURE 8A, FIGURE 8B and FIGURE 8C show the examples of HEK 293, CHO and CHW cell lines co-expressing adrenergic receptor β 2AR and arrestin fusion proteins of β -

galactosidase mutants. The β -galactosidase activity was used to monitor agonist-induced interaction of β 2AR and arrestin proteins.

FIGURE 9. Beta-gal complementation can be used to monitor β 2 adrenergic receptor homo-dimerization. FIGURE 9A shows β -galactosidase activity in HEK 293 clones co-expressing β 2AR- β gal $\Delta\alpha$ and β 2AR- β gal $\Delta\omega$. FIGURE 9B shows a cAMP response to agonist (-)-isoproterenol in HEK 293 clones co-expressing β 2AR- β gal $\Delta\alpha$ and β 2AR- β gal $\Delta\omega$. HEK293 parental cells were included in the assays as negative controls.

FIGURE 10A. pICAST ALC: Vector for expression of β -gal $\Delta\alpha$ as a C-terminal fusion to the target protein. This construct contains the following features: MCS, multiple cloning site for cloning the target protein in frame with the β -gal $\Delta\alpha$; GS Linker, (GGGGS) n ; NeoR, neomycin resistance gene; IRES, internal ribosome entry site; ColE1ori, origin of replication for growth in E. coli; 5'MoMuLV LTR and 3'MoMuLV LTR, viral promoter and polyadenylation signals from the Moloney Murine leukemia virus.

FIGURE 10B. Nucleotide sequence for pICAST ALC.

FIGURE 11A. pICAST ALN: Vector for expression of β -gal $\Delta\alpha$ as an N-terminal fusion to the target protein. This construct contains the following features: MCS, multiple cloning site for cloning the target protein in frame with the β -gal $\Delta\alpha$; GS Linker, (GGGGS) n ; NeoR, neomycin resistance gene; IRES, internal ribosome entry site; ColE1ori, origin of replication for growth in E. coli;

5'MoMuLV LTR and 3'MoMuLV LTR, viral promoter and polyadenylation signals from the Moloney Murine leukemia virus.

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A3

FIGURE 11B. Nucleotide sequence for pICAST ALN.

FIGURE 12A. pICAST OMC: Vector for expression of β -gal $\Delta\omega$ as a C-

5 terminal fusion to the target protein. This construct contains the following features: MCS, multiple cloning site for cloning the target protein in frame with the β -gal $\Delta\omega$; GS Linker, (GGGGS) $_n$; Hygro, hygromycin resistance gene; IRES, internal ribosome entry site; ColE1ori, origin of replication for growth in E. coli; 5'MoMuLV LTR and 3'MoMuLV LTR, viral promoter and polyadenylation signals from the Moloney Murine leukemia virus.

Sub
A4

FIGURE 12B. Nucleotide sequence for pICAST OMC.

FIGURE 13A. pICAST OMN: Vector for expression of β -gal $\Delta\omega$ as an N-terminal fusion to the target protein. This construct contains the following

features: MCS, multiple cloning site for cloning the target protein in frame with the β -gal $\Delta\omega$; GS Linker, (GGGGS) $_n$; Hygro, hygromycin resistance gene; IRES, internal ribosome entry site; ColE1ori, origin of replication for growth in E. coli; 5'MoMuLV LTR and 3'MoMuLV LTR, viral promoter and polyadenylation signals from the Moloney Murine leukemia virus.

Sub
A5

FIGURE 13B. Nucleotide sequence for pICAST OMN.

20 FIGURE 14. pICAST ALC β Arr2: Vector for expression of β -gal $\Delta\alpha$ as a C-terminal fusion to β -arrestin-2. The coding sequence of human β -arrestin-2 (Genebank Accession Number: NM_004313) was cloned in frame to β -gal $\Delta\alpha$ in a

pICAST ALC vector.

FIGURE 15. pICAST OMC β Arr2: Vector for expression of β -gal $\Delta\omega$ as a C-terminal fusion to β -arrestin-2. The coding sequence of human β -arrestin-2 (Genebank Accession Number: NM_004313) was cloned in frame to β -gal $\Delta\omega$ in a pICAST OMC vector.

FIGURE 16. pICAST ALC β Arr1: Vector for expression of β -gal $\Delta\alpha$ as a C-terminal fusion to β -arrestin-1. The coding sequence of human β -arrestin-1 (Genebank Accession Number: NM_004041) was cloned in frame to β -gal $\Delta\alpha$ in a pICAST ALC vector.

FIGURE 17. pICAST OMC β Arr1: Vector for expression of β -gal $\Delta\omega$ as a C-terminal fusion to β -arrestin-1. The coding sequence of human β -arrestin-1 (Genebank Accession Number: NM_004041) was cloned in frame to β -gal $\Delta\omega$ in a pICAST OMC vector.

FIGURE 18. pICAST ALC β 2AR: Vector for expression of β -gal $\Delta\alpha$ as a C-terminal fusion to β 2 Adrenergic Receptor. The coding sequence of human β 2 Adrenergic Receptor (Genebank Accession Number: NM_000024) was cloned in frame to β -gal $\Delta\alpha$ in a pICAST ALC vector.

FIGURE 19. pICAST OMC β 2AR: Vector for expression of β -gal $\Delta\omega$ as a C-terminal fusion β 2 Adrenergic Receptor. The coding sequence of human β 2 Adrenergic Receptor (Genebank Accession Number: NM_000024) was cloned in frame to β -gal $\Delta\omega$ in a pICAST OMC vector.

FIGURE 20. pICAST ALC A2aR: Vector for expression of β -gal $\Delta\alpha$ as a C-terminal fusion to Adenosine 2a Receptor. The coding sequence of human Adenosine 2a Receptor (Genebank Accession Number: NM_000675) was cloned in frame to β -gal $\Delta\alpha$ in a pICAST ALC vector.

5 FIGURE 21. pICAST OMC A2aR: Vector for expression of β -gal $\Delta\omega$ as a C-terminal fusion to Adenosine 2a Receptor. The coding sequence of human Adenosine 2a Receptor (Genebank Accession Number: NM_000675) was cloned in frame to β -gal $\Delta\omega$ in a pICAST OMC vector.

10 FIGURE 22. pICAST ALC D1: Vector for expression of β -gal $\Delta\alpha$ as a C-terminal fusion to Dopamine D1 Receptor. The coding sequence of human Dopamine D1 Receptor (Genebank Accession Number: X58987) was cloned in frame to β -gal $\Delta\alpha$ in a pICAST ALC vector.

15 FIGURE 23. A schematic depicting use of the complementation technology in the method of the invention. FIGURE 23 shows two inactive mutant reporter enzymes that become active when the corresponding fusion partners, GPCR and β -arrestin interact.

20 FIGURE 24. Vector for expression of a GPCR with inserted seronine/threonine amino acid sequences as a fusion with β -gal $\Delta\alpha$. The open reading frame of a known or orphan GPCR is engineered to contain additional seronine/threonine sequences, such as SSS (seronine, seronine, seronine), within the C-terminal tail. The engineered GPCR is cloned in frame with β -gal $\Delta\alpha$ in a pICAST ALC vector. The pICAST ALC vector contains the following features:

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MCS, multiple cloning site for cloning the target protein in frame with the β -gal $\Delta\alpha$; GS Linker, (GGGGS) $_n$; NeoR, neomycin resistance gene; IRES, internal ribosome entry site; ColE1ori, origin of replication for growth in E. coli; 5'MoMuLV LTR and 3'MoMuLV LTR, viral promotor and polyadenylation signals from the

5 Moloney Murine leukemia virus.

FIGURE 25. Vector for expression of mutant (R170E) β -arrestin2 as a fusion with β -gal $\Delta\omega$. The open reading frame of β -arrestin2 is engineered to contain a point mutation that converts arginine 170 to a glutamate. The mutant β -arrestin2 is cloned in frame with β -gal $\Delta\omega$ in a pICAST OMC vector. The pICAST

10 OMC vector contains the following features: MCS, multiple cloning site for cloning the target protein in frame with the β -gal $\Delta\alpha$; GS Linker, (GGGGS) $_n$; Hygro, hygromycin resistance gene; IRES, internal ribosome entry site; ColE1ori, origin of replication for growth in E. coli; 5'MoMuLV LTR and 3'MoMuLV LTR, viral promotor and polyadenylation signals from the Moloney Murine leukemia

15 virus.

FIGURE 26. Phosphorylation insensitive Mutant R170E β -Arrestin2 $\Delta\omega$ binds to β 2AR $\Delta\alpha$ in Response to Agonist Activation. A parental β 2AR $\Delta\alpha$ C2 cell line was transduced with the Mutant R170E β -Arrestin2 $\Delta\omega$ construct. Clonal populations co-expressing the two constructions were plated at 10,000 cells/well in

20 96 well plates and treated with 10 μ M (-)isoproterenol, 0.3mM ascorbic acid for the indicated time period. β -galactosidase activity was measured by addition of Tropix Gal-ScreenTM assay system substrate (Applied Biosystems) and luminescence was measured using a Tropix TR717TM luminometer (Applied Biosystems). Treatments

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FIG 27.25T5.250

were performed in triplicate. For comparison, a clonal cell line (43-8) co-expressing $\beta 2AR\Delta\alpha$ and wild-type β -Arrestin2 $\Delta\omega$ was also plated at 10,000 cells/well and given the same agonist treatment regimen. Minutes of (-)isoproterenol treatment is shown on the X-axis and β -galactosidase activity indicated by relative light units (RLU) is shown on the Y-axis.

FIGURE 27. GPCR dimerization measured by β -galactosidase complementation. A schematic depicting the utilization of the invention for monitoring GPCR homo- or hetero- dimerization. One GPCR is fused to one complement enzyme fragment, while the second GPCR is fused to the second complement enzyme fragment. Interaction of the two GPCRs is monitored by complementation of the enzyme fragments to produce an active enzyme complex (i.e., β -galactosidase activity). GPCR homo- or hetero- dimerization can be monitored in the absence or presence of ligand, agonists, inverse agonists or antagonists.

FIGURE 28. Ligand fishing for orphan receptors by β -galactosidase mutant complementation in ICAST™ system. A schematic depicting the utilization of the invention for ligand fishing and agonist/antagonist screening for orphan GPCRs. As an example, a test cell expressing two β -gal fusion proteins, $GPCR_{\text{orphan}}\Delta\alpha$ and Arrestin- $\Delta\omega$, is subjected to treatments with samples from natural or synthetic compound libraries, or from tissue extracts, or from conditioned media of cultured cells. An increased β -gal activity after treatment indicates the activation of the orphan receptor by a ligand in the testing sample. The readout of increased β -gal activity reflects the interaction of an activated

GPCR orphan receptor with a β -arrestin. Therefore, a cognate or a surrogate ligand for the testing receptor is identified.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

5 The present invention provides a method to interrogate GPCR function and pathways. The G-protein-coupled superfamily continues to expand rapidly as new receptors are discovered through automated sequencing of cDNA libraries or genomic DNA. It is estimated that several thousand GPCRs may exist in the human genome. Only a portion have been cloned and even fewer have been
10 associated with ligands. The means by which these, or newly discovered orphan receptors, will be associated with their cognate ligands and physiological functions represents a major challenge to biological and biomedical research. The identification of an orphan receptor generally requires an individualized assay and a guess as to its function. The present invention involves the interrogation of
15 GPCR function by monitoring the activation of the receptor using activation dependent protein-protein interactions between the test GPCR or orphan receptor and a β -arrestin. The specific protein-protein interactions are measured using the mutant enzyme complementation technology disclosed herein. This assay system eliminates the prerequisite guessing because it can be performed with and without
20 prior knowledge of other signaling events. It is sensitive, rapid and easily performed and is applicable to nearly all GPCRs because the majority of these receptors desensitize by a common mechanism.

 The present invention provides a complete assay system for monitoring

protein-protein interactions in GPCR pathways. The invention employs the complementation technology, ICAST™ (Intercistronic Complementation Analysis Screening Technology as disclosed in pending U.S. patent application serial no. 053,614, filed April 1, 1998, the entire contents of which are incorporated herein
5 by reference). The ICAST™ technology involves the use of two mutant forms of a reporter enzyme fused to proteins of interest. When the proteins of interest do not interact, the reporter enzyme remains inactive. When the proteins of interest do interact, the reporter enzyme mutants come together and form an active enzyme. According to an embodiment of the invention, the activity of β -galactosidase may
10 be detected with the Gal-Screen™ assay system developed by Advanced Discovery Sciences™, which involves the use of Galacton-*Star*®, an ultrasensitive chemiluminescent substrate. The Gal-Screen™ assay system and the Galacton-*Star*® chemiluminescent substrate are disclosed in U.S. Patent Nos. 5,851,771; 5,538,847; 5,326,882; 5,145,772; 4,978,614; and 4,931,569, the contents of which
15 are incorporated herein by reference in their entirety. The invention provides an array of assays, including GPCR binding assays, that can be achieved directly within the cellular environment in a rapid, non-radioactive assay format. The methods of the invention are an advancement over the invention disclosed in U.S. Patent Nos. 5,891,646 and 6,110,693 and the method disclosed in Angers et al.,
20 supra., which rely on microscopic imaging or spectrometry of GPCR components as fusion with Green-fluorescent-protein. The imaging technique disclosed in U.S. Patent Nos. 5,891,646 and 6,110,693 and spectrometry-based technique in Angers et al. are limited by low-throughput and lack of thorough quantification.

The assay system of the invention combined with Advanced Discovery Sciences™ technologies provide highly sensitive cell-based methods for interrogating GPCR pathways which are amenable to high-throughput screening (HTS). Among some of the technologies developed by Advanced Discovery

5 Sciences™ that may be used with the present invention are the Gal-Screen™ assay system (discussed above) and the cAMP-Screen™ immunoassay system. The cAMP-Screen™ immunoassay system provides ultrasensitive determination of cAMP levels in cell lysates. The cAMP-Screen™ assay utilizes the high-sensitivity chemiluminescent alkaline phosphatase (AP) substrate CSPD® (disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro) tricyclo 3.3.1.1.^{3,7}} decan-4-yl phenyl phosphate) with Sapphire-II™ luminescence enhancer.

Unlike yeast-based-two-hybrid assays used to monitor protein/protein interactions in high-throughput assays, the present invention (1) is applicable to a variety of cells including mammalian cells, plant cells, protozoa cells such as E. coli and cells of invertebrate origin such as yeast, slime mold (*Dictyostelium*) and insects; (2) detects interactions at the membrane at the site of the receptor target or in the cytosol at the site of downstream target proteins rather than a limited cellular localization, i.e., nucleus; and (3) does not rely on indirect read-outs such as transcriptional activation. The present invention thus provides assays with greater

15

20 physiological relevance and fewer false positives.

The present inventors have developed modifications to the embodiment disclosed in U.S. patent application serial no. 053,614 described above in order to enhance the sensitivity of the inventive GPCR assay. According to an

embodiment, the invention incorporates the use of serine/threonine clusters to enhance and prolong the interaction of GPCR with arrestin in order to make the detection more robust. The clusters can be utilized for orphan receptors or known GPCRs, which do not have this sequence motif. By adding this sequence to the C-terminal tail of the receptor, the activation of the receptor can be detected more readily by readouts of arrestin binding to GPCR, i.e., β -galactosidase complementation from fusion proteins of target proteins with β -galactosidase mutants.

According to another embodiment, the invention incorporates the use of arrestin point mutations to bypass the requirement of phosphorylation, by the action of specific GRK, on the C-terminal tail or intracellular loops of GPCR upon activation. The applications include i) wherein the cognate GRK for a particular GPCR or orphan receptor is unknown; and ii) wherein the specific GRK for the receptor of interest (or under test) may not be present or may have low activity in the host cell that is used for receptor activation assay.

According to another embodiment, the invention incorporates the use of a super arrestin to increase the binding efficiency of arrestin to an activated GPCR and to stabilize the GPCR/arrestin complex during GPCR desensitization. This application can be used to increase the robustness of ICAST/GPCR applications in cases where the GPCR is normally resensitized rapidly post desensitization.

Each of these methodologies is discussed below.

The invention will now be described in the following non-limiting examples.

EXAMPLE:

According to an embodiment of the invention, GPCR activation is measured through monitoring the binding of arrestin to ligand-activated GPCR. In this assay system, a GPCR, e.g., β -adrenergic receptor (β 2AR), and an arrestin, e.g., β -arrestin, are co-expressed in the same cell as fusion proteins with mutant forms of a reporter enzyme, e.g., β -galactosidase (β -gal). As illustrated in Figure 23, the β 2AR is expressed as a fusion protein with $\Delta\alpha$ form of β -gal mutant (β 2AR $\Delta\alpha$) and the β -arrestin as a fusion protein with the $\Delta\omega$ form of β -gal mutant (β -Arr $\Delta\omega$). The two fusion proteins, which at first exist in a resting (or un-stimulated) cell in separate compartments, i.e., the membrane for GPCR and the cytosol for arrestin, cannot form an active β -galactosidase enzyme. When such a cell is treated with an agonist or a ligand, the ligand-occupied and activated receptor becomes a high affinity binding site for arrestin. The interaction between an activated GPCR, β 2AR $\Delta\alpha$, and arrestin, β -Arr $\Delta\omega$, drives the β -gal mutant complementation. The enzyme activity can be measured by using an enzyme substrate, which upon cleavage releases a product measurable by colorimetry, fluorescence, or chemiluminescence (e.g., the Gal-Screen™ assay system).

Experiment protocol-

1. In the first step, the expression vectors for β 2AR $\Delta\alpha$ and β Arr2 $\Delta\omega$ were engineered in selectable retroviral vectors pICAST ALC, as described in Figure 18 and pICAST OMC, as described in Figure 15.

2. In the second step, the two expression constructs were transduced into either C2C12 myoblast cells, or other mammalian cell lines, such as COS-7, CHO, A431, HEK 293, and CHW. Following selection with antibiotic drugs, stable clones expressing both fusion proteins at appropriate levels were selected.

3. In the last step, the cells expressing both $\beta 2AR\Delta\alpha$ and $\beta Arr2\Delta\omega$ were tested for response by agonist/ligand stimulated β -galactosidase activity. Triplicate samples of cells were plated at 10,000 cells in 100 microliter volume into a well of 96-well culture plate. Cells were cultured for 24 hours before assay. For agonist assay (Figures 3 and 4), cells were treated with variable concentrations of agonist, for example, (-) isoproterenol, procaterol, dobutamine, terbutaline or L-L-phenylephrine for 60 min at 37° C. The induced β -galactosidase activity was measured by addition of Tropix Gal-Screen™ assay system substrate (Applied Biosystems) and luminescence measured in a Tropix TR717™ luminometer (Applied Biosystems). For antagonist assay (Figure 5), cells were pre-incubated for 10 min in fresh medium without serum in the presence of ICI-118,551 or propranolol followed by addition of 10 micro molar (-) isoproterenol.

Serine/Threonine Cluster Strategy

Background

Based on structure-function relationship studies on β -arrestins, a large region within the amino-terminal half of β -arrestins (termed the activation-recognition domain) recognizes the agonist-activated state of GPCRs. This region of β -arrestin also contains a small positively charged domain (approximately 20

amino acids with net charge +7) called the phosphorylation-recognition domain, which appears to interact with the GRK-phosphorylated carboxyl termini of GPCRs.

GPCRs can be divided into two classes based on their affinities for β -arrestins. Oakley et al., "Association of β -Arrestin with G Protein-Coupled Receptors During Clathrin-Mediated Endocytosis Dictates the Profile of Receptor Resensitization." J. Biol. Chem., 274(45):32248-32257 (1999). The molecular determinants underlying this classification appear to reside in specific serine or threonine residues located in the carboxyl-terminal tail of the receptor. The receptor class that contains serine/threonine clusters (defined as serine or threonine residues occupying three consecutive or three out of four positions) in the carboxyl-termini binds β -arrestin with high affinity upon activation and phosphorylation and remains bound with β -arrestin even after receptor internalization, whereas the receptor class that contains only scattered serine and threonine residues in the carboxy-terminal tail binds β -arrestins with less affinity and disassociates from the β -arrestin upon internalization. Several known GPCRs, such as vasopressin V2 receptor (Oakley, et al.), neurotensin receptor 1 and angiotensin II receptor type 1A (Zhang, et al., "Cellular Trafficking of G Protein-Coupled Receptor/ β -Arrestin Endocytic Complexes." J. Biol. Chem., 274(16):10999-11006 (1999)), which possess one or more of such serine/threonine clusters in their carboxyl-termini, were shown to bind β -arrestins with high affinity.

EXAMPLE

According to an embodiment of the invention, a serine/threonine cluster strategy is used to facilitate screening assays for orphan receptors that do not possess this structural motif of their own. The orphan receptors are easily classified by sequence alignment. Orphan receptors lacking the serine/threonine clusters are each cloned into an expression vector that is modified to introduce one or more serine/threonine cluster(s) to the carboxyl-terminal tail of the receptor (FIGURE 24). The serine/threonine clusters enhance the receptor activation dependent interaction between the activated and phosphorylated receptor (negative charges) and β -arrestin (positive charges in the phosphorylation-recognition domain) through strong ionic interactions, thus prolonging interaction between the receptor and arrestin. The modification of the orphan receptor tail thus makes detection of receptor activation more robust.

Experiment protocol -

1. In a first step, the open-reading-frame (ORF) of an orphan receptor, which lacks the serine/threonine clusters, is cloned into a modified expression vector such as pICAST ALC described in Figure 10A. The modified pICAST ALC includes coding sequences for one or more sets of serine/threonine clusters (for example, SSS or SST) located downstream from the insert of the ORF of an orphan receptor (FIGURE 24).

2. In a second step, chimeric orphan receptor, $\text{ORF}_{\text{orphan R}}-(\text{SSS})_n-\Delta\alpha$, is co-

expressed in a mammalian cell with a β -arrestin chimera, such as β Arr2 $\Delta\omega$ described in Figure 15.

3. In a third step, the cell is treated with an agonist or a ligand and the activated receptor with phosphorylated serine cluster(s) binds the β -arrestin with high affinity producing strong signals in readouts of β -gal complementation.

This assay, which provides a means for sensitive measurement of functional activation of the orphan receptors, can be used to screen for natural or surrogate ligands for orphan receptors, a process called de-orphaning or target discovery for new GPCRs (FIGURE 28). Furthermore, this assay is also useful in screening for potential agonists and antagonists for lead discovery of GPCRs.

Enhanced Binding of Arrestin in the Presence and in the Absence of GPCR

Phosphorylation

Background

Six different classes of G-protein coupled receptor kinases (GRKs) have been identified and each of these has been reported to be expressed as multiple splice variants. Krupnick et al., "The role of receptor kinases and arrestins in G protein-coupled receptor regulation." Ann. Rev. Pharmacol. Toxicol., 38:289-319 (1998). Although many cell lines express a variety of GRKs, the specific GRK required for phosphorylation of a given GPCR may not always be present in the cell line used for recombinant GPCR and arrestin expression. This is particularly an issue for applications using orphan receptors, in which case the cognate GRK will likely be unknown. In other cases, the cell line used for recombinant

expression work may have the required GRK, but may express the GRK at low levels. In order to bypass such caveats, genetically modified arrestins that bind specifically to activated GPCRs, but without the requirement of GRK phosphorylation are employed.

5 Mutagenesis studies on arrestins demonstrate that point mutations in the phosphorylation-recognition domain, particularly mutations converting Arg175 (of visual arrestin) to an oppositely charged residue such as glutamate (R175E mutation), result in an arrestin which specifically binds to activated GPCRs, but does so without the requirement for phosphorylation.

10 Numerous observations have led to the hypothesis that arrestin exists in an inactive state that has a low affinity for GPCRs. Once a GPCR is both activated and phosphorylated, the phosphorylated region of the GPCR C-terminus interacts with the phosphorylation-recognition domain of arrestin causing the arrestin to change conformations allowing the activation-recognition region to be exposed for
15 binding to the activated/ phosphorylated receptor. Vishnivetskiy et al., "How does arrestin respond to the phosphorylated state of rhodopsin?" J. Biol. Chem., 274(17):11451-11454 (1999); Gurevich et al., "Arrestin interactions with G protein-coupled receptors. Direct binding studies of wild-type and mutant arrestins with rhodopsin, beta 2-adrenergic and m2 muscarinic cholinergic receptors." J.
20 Biol. Chem., 270(2):720-731, (1995); Gurevich et al., "Mechanism of phosphorylation-recognition by visual arrestin and the transition of arrestin into a high affinity binding site." Mol. Pharmacol., 51(1):161-169 (1997); Kovoor et al., "Targeted construction of phosphorylation-independent beta-arrestin mutants with

constitutive activity in cells.” J. Biol. Chem., 274(11):6831-6834 (1999). In summary, binding studies of single mutation, double mutation, deletion, and chimerical arrestins with inactive, inactive and phosphorylated, activated but not phosphorylated, or activated and phosphorylated visual or non-visual GPCRs all support this model.

EXAMPLE

A phosphorylation insensitive mutant of arrestin fused to mutant reporter protein can be produced that will bind to activated GPCRs in a phosphorylation independent manner. As proof of concept, a point mutation for β -arrestin2, R170E β -arrestin2, has been produced and its interaction with β 2AR has been analyzed in accordance with the invention.

Experimental protocol:

1) In the first step, β -arrestin2 was mutated such that Arg170 was converted to Glu. This mutation is equivalent to the R175E mutation of visual arrestin. The mutant β -arrestin2 open reading frame was cloned in frame with $\Delta\omega$ - β -galactosidase in the pICAST OMC expression vector to produce a modified expression vector R170E β -arrestin2 (FIGURE 25).

2) In the second step, the R170E β -arrestin2 expression construct was transduced into a C2C12 myoblast cell line that had been engineered to express β 2AR as a fusion to $\Delta\alpha$ - β -galactosidase as described in Figure 18 of U.S.

Application Serial No. 09/654,499. Following selection with antibiotic drugs, a

population of clones expressing both fusion proteins was obtained.

- 3) In the last step, this population of cells expressing both R170E β -arrestin2 $\Delta\omega$ and β 2AR $\Delta\alpha$ were tested for response by agonist/ligand stimulated β -galactosidase activity as demonstrated in FIGURE 26. The C2C12 clone 43-8 co-expressing β 2AR $\Delta\alpha$ and wild-type β -arrestin2 $\Delta\omega$ (FIGURE 26) was used as reference control. Triplicate samples of cells were plated at 10,000 cells in 100 microliter volume into wells of a 96-well culture plate. Cells were cultured for 24 hours before assay. For agonist assay as in FIGURE 26, cells were treated with 10 μ m (-)isoproterenol stabilized with 0.3mM ascorbic acid 37° C for 0, 5, 10, 15, 30, 45 or 60 minutes. The induced β -galactosidase activity was measured by addition of Tropix Gal-Screen™ assay system substrate (Applied Biosystems) and luminescence measured in a Tropix TR717™ luminometer (Applied Biosystems). As shown in Figure 26, the mutant arrestin interacts with β 2AR in an agonist-dependent manner and was comparable with that of wild-type arrestin.
- 4) To expand the application of phosphorylation-insensitive arrestin, cell lines such as C2C12, CHO or HEK 293, are developed that express the R170E β -arrestin2 $\Delta\omega$ construction. These cell lines can be used to transduce orphan or known GPCRs as fusions with $\Delta\alpha$ - β -galactosidase in order to develop cell lines for agonist and antagonist screening and

Development of Super Arrestins:

Background

Attenuation of GPCR signaling by the arrestin pathway serves to ensure that a cell or organism does not over-react to a stimulus. At the same time, the
5 arrestin pathway often serves to recycle the GPCR such that it can be temporarily inactivated but then quickly resensitized to allow for sensitivity to new stimuli.

The down-regulation process involves phosphorylation of the receptor, binding to arrestin and endocytosis. Following endocytosis of the desensitized receptor, the receptor is either degraded in lysosomes or resensitized and sent back to the
10 membrane. Resensitization involves release of arrestin from the receptor, dephosphorylation and cycling back to the membrane. The actual route a GPCR follows upon activation depends on its biological function and the needs of the organism. Because of these diverse pathways that may be required of the down-regulation pathway, arrestin affinities for activated GPCRs vary from receptor to
15 receptor. It would thus be very advantageous to engineer super arrestins that have a higher affinity and avidity for activated GPCRs than what nature has provided.

Although mutational, deletion and chimerical studies of arrestins have focused on understanding regulatory switches in the molecule that respond to GPCR phosphorylation states, several of these altered recombinant forms of
20 arrestin have resulted in molecules with enhanced binding to activated, phosphorylated GPCRs. Conversion of Arg175 to histidine, tyrosine, phenylalanine or threonine results in significantly higher amounts of binding to phosphorylated, activated rhodopsin than wild-type arrestin or R175E arrestin,

although these mutations result in less binding to activated, non-phosphorylated receptor. Gurevich et al. (1997). In addition, conversion of Valine 170 to alanine increased the constitutive affect of the R175E mutation, but also nearly doubled the amount of interaction of wild-type arrestin with activated, phosphorylated rhodopsin. Gurevich et al. (1997).

Truncation of β -arrestin1 at amino acid 382 has been reported to enhance binding of both R169E (equivalent to arrestin R175E) and wild-type β -arrestin1 to activated or activated and phosphorylated receptor, respectively. Kovoor et al. Chimerical arrestins in which functional regions of visual arrestin were swapped with those of β -arrestin1 have been reported to be altered in binding affinity to activated, phosphorylated GPCRs. Gurevich et al. (1995). Several of these chimeras, such as β -arrestin1 containing the visual arrestin extreme N-terminus, show increased specific binding to phosphorylated activated GPCRs compared to wild-type β -arrestin1 (Gurevich et al. (1995)). Modifications that enhance arrestin affinity for the activated GPCR such as described above, whether phosphorylated or non-phosphorylated, could also enhance signal to noise of β -galactosidase activity since the arrestin/GPCR complex is stabilized and/or more long-lived. The use of mutant arrestins with higher activated-GPCR affinity would improve the inventive technology for GPCR targets, without compromising receptor/ligand biology.

In addition, this “super arrestin” approach can be combined with the use of arrestin point mutations to provide a stronger signal to noise with or without GRK requirements.

EXAMPLE

An arrestin mutant fused to mutant reporter protein can be produced to enhance binding of the arrestin to an activated GPCR to enhance sensitivity of detection.

5 Experiment protocol -

- 1) In the first step, mutant β -arrestin2 constructions will be generated which include R170E/T/Y/or H, V165A, substitution of a.a. 1-43 with a.a. 1-47 of visual arrestin, or deletion of the C-terminal and combinations of these alterations. The mutant β -arrestin2 open reading frames will be cloned in frame with $\Delta\omega$ - β -galactosidase in the pICAST OMC expression vector similar to cloning of the R170E β -arrestin2 mutation shown in FIGURE 25.
- 2) In the second step, mutant expression constructs will be transduced into a C2C12 myoblast cell line that has been engineered to express β 2AR as a fusion to $\Delta\alpha$ - β -galactosidase. Following selection with antibiotic drugs, a population of clones expressing both fusion proteins will be obtained. Wild type and R170E β -arrestin2 constructions will be transduced to generate control, reference clonal populations.
- 3) In the third step, populations of cells expressing both β -arrestin2 $\Delta\omega$ (mutant or wild type) and β 2AR $\Delta\alpha$ will be tested for response by agonist/ligand stimulated β -galactosidase activity.
- 4) In the next step, mutant (super) β -arrestin2 $\Delta\omega$ constructions that show a significantly higher signal to noise ratio in the agonist assay compared with wild-type β -arrestin2 $\Delta\omega$ will be chosen. These constructions will be used to develop

stable cell lines expressing the “super” β -arrestin2 $\Delta\omega$ that can be used for transducing in known or orphan GPCRs. Use of a super β -arrestin2 $\Delta\omega$ could increase the signal to noise of ICAST/GPCR applications allowing improved screening capabilities for lead and ligand discovery.

5 Super Arrestin is used to increase the binding efficiency of arrestin to an activated GPCR and to stabilize the GPCR/arrestin complex during GPCR desensitization. This application can be used to increase the robustness of ICAST/GPCR applications in cases where the GPCR is normally resensitized rapidly post desensitization.

10 The assays of this invention, and their application and preparation have been described both generically, and by specific example. The examples are not intended as limiting. Other substituent identities, characteristics and assays will occur to those of ordinary skill in the art, without the exercise of inventive faculty. Such modifications remain within the scope of the invention, unless excluded by
15 the express recitation of the claims advanced below.